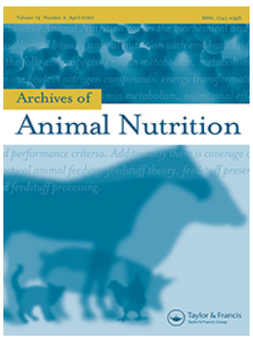

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Formation of volatile organic compounds during the fermentation of maize as affected by sealing time and silage additive use

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ABSTRACT

The study investigated the production of volatile organic compounds during the fermentation of maize containing 26.8% dry matter (DM). Forage was ensiled without additive or treated with 2 ml/kg of a chemical silage additive (SA) containing per litre 257 g sodium benzoate, 134 g potassium sorbate and 57 g ammonium propionate, and either sealed immediately or with a delay of 24 h. During the fermentation process, DM-losses, fermentation pattern (including ethyl lactate [EL] and ethyl acetate [EA]) and yeast numbers were determined. Delayed sealing and no SA resulted in highest DM losses with significant interactions between sealing time (ST) and SA on all sampling days ($p < 0.001$). The effects on organic acid production were variable depending on storage length. Ethanol production was affected by ST and SA, but promptly sealed silage treated with SA had consistently the lowest concentrations. Higher ethanol content during fermentation was associated with higher DM losses, as reflected by a strongly linear, positive relationship ($R^2 = 0.70$, $p < 0.001$). Compared with promptly sealed silage, the counts of yeasts were higher after delayed sealing during the first 7 d of storage ($p < 0.001$). Moreover, SA reduced yeast numbers compared with untreated silage ($p < 0.01$). EL concentrations increased throughout storage, whereas EA acetate accumulation was very rapid and intense already during the early stages of fermentation and peaked on d 34. The differences in concentrations and accumulation pattern between EL and EA, especially during the early fermentation phases, make evident that their synthesis was facilitated by different pathways and reactions, respectively.

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Ensiling; esters; ethanol; fermentation products; maize; silage additives; volatile compounds; yeasts

1. Introduction

Incidence and concentration of volatile organic compounds (VOC) may vary significantly depending on crop, storage conditions and use of silage additive (SA) (Weiss et al. 2016; Brüning et al. 2018a). The major volatile fermentation acids, e.g. acetic, propionic and butyric acids are produced by heterofermentative lactic acid bacteria (LAB), enterobacteria or clostridia (McDonald et al. 1991; Pahlow et al. 2003). However, the most prominent alcohols in fermented forages, ethanol and propanol, are end-products of

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metabolic pathways of heterofermentative LAB and yeasts (McDonald et al. 1991; Driehuis and van Wikselaar 2000; Krooneman et al. 2002). Other VOC, such as esters and aldehydes, may be formed by different microbial populations, including yeasts occurring in silage (Kruis et al. 2017) and LAB (Liu and Siezen 2006), but also abiotically by chemical synthesis (Peter and Vollhardt 1988). In different investigations, VOC (comprising esters and alcohols) were analysed but detected concentrations differed between crops and experimental conditions (Weiss 2017).

The occurrence of VOC in different silage types has recently attracted significant attention due to their potential to contribute to air pollution (Howard et al. 2010; Hafner et al. 2010, 2012; Malkina et al. 2011). Furthermore, due to their chemical structure and the possible negative effects of an odd (atypical) smell, VOC may impair feed intake and performance as well as metabolism of ruminants, including dairy cows (Krizsan et al. 2007; Raun and Kristensen 2010; Gerlach et al. 2013). For most of these compounds, which are less frequently analysed, the knowledge on their mode of action in the animal is limited (Brüning et al. 2018b).

Because particular silages with markedly increased ethanol content contained also significant concentrations of other VOC, especially esters were considered as indicator substances for the total VOC production (Weiss et al. 2016; Weiss 2017). Among other factors, delayed sealing and use of SA affected the fermentation pattern and VOC formation in silages made from maize, grass and sorghum (Weiss et al. 2016; Weiss 2017; Brüning et al. 2018a). According to Weiss et al. (2016), salts of sorbic, benzoic or propionic acids or their mixtures have been shown to be the most promising additives to control VOC production in maize silage.

Due to the lack of data on the formation of esters during the course of fermentation, the aim of the present study was to examine the accumulation of ethyl esters and their corresponding reactants ethanol, acetic acid and lactic acid, depending on storage length. The experimental design was based on the hypothesis that VOC formation, yeast development and dry matter (DM) losses are strongly affected by delayed sealing and the use of fungal inhibitors.

2. Materials and methods

2.1. Experimental design

A 6-ha plot of half-bog soil (hypereutric chernic gleysol) belonging to a dairy farm in Trebbin (52°19´55131"; 13°24`46269") was used for cultivation of the late-maturing (FAO number: 320) forage maize hybrid PR38Y34 (Pioneer Hi-Bred Northern Europe Service Division GmbH, Buxtehude, Germany). Maize was planted on 26 April 2015 at a density of 83,000 plants/ha and received a total of 120 kg nitrogen per ha exclusively from cattle slurry. The forage was harvested at the early dough stage on 8 September 2015 by a Krone Big X chopper, which was set at a theoretical chop length of 10 mm. Chopped maize was thoroughly mixed and thereafter divided into two piles from which samples were taken from different locations to determine chemical composition and fungal counts (Table 1). One forage pile was left untreated (CON), whereas the material of another pile was spread out in a thin layer and was treated with a chemical SA (composed per litre of 257 g sodium benzoate, 134 g potassium sorbate and 57 g ammonium

Table 1. Chemical composition and microbial counts of fresh maize before silo filling (means).

	n	Without additive	With additive[†]
Dry matter [g/kg]	3	26.8	27.3
Crude protein [g/kg DM]	2	79.0	77.6
Ether extract [g/kg DM]	2	25.6	25.6
Crude fibre [g/kg DM]	2	213	211
Crude ash [g/kg DM]	2	45.9	46.4
NDFom* [g/kg DM]	2	453	446
ADFom[#] [g/kg DM]	2	241	235
ADL[+] [g/kg DM]	2	31.6	31.6
Water-soluble carbohydrates [g/kg DM]	2	158	150
Buffering capacity [g lactic acid/kg DM]	2	27.4	28.9
Nitrate [g/kg DM]	2	1.6	1.7
Yeast count [$\log_{10}$ cfu/fresh matter]	6	4.4	4.3
Mould count [$\log_{10}$ cfu/fresh matter]	6	5.5	5.3

*NDFom, neutral detergent fibre, expressed exclusive of residual ash; [#]ADFom, acid detergent fibre, expressed exclusive of residual ash; [+]ADL, acid detergent lignin; [†]composed per litre of 257 g sodium benzoate, 134 g potassium sorbate, 57 g ammonium propionate, applied at 2 ml/kg.

propionate) at a rate of 2 ml/kg. In order to ensure homogeneous application, the additive was diluted with tap water to 10 ml/kg application volume, manually applied by using a garden sprayer and thoroughly mixed. After packing into 1.5-l glass jars (Weck, Öfingen, Germany) at a density of 198 ± 2 kg DM/m^3, the experimental silos were either immediately sealed (prompt) or sealed with a delay of 24 h (delay). For each of the four tested treatments CON, CON_delay, SA, SA_delay, three replicate silages per treatment and sampling time (after 3, 7, 16, 34, 62 and 142 d of storage) were produced, totalling to 72 experimental silages. All silos were kept at 20–22°C throughout the entire storage length.

2.2. Determination of dry matter

The content of DM in fresh forage and silage was determined after drying at 60°C until constant weight, followed by drying at 105°C for 3 h (Weissbach and Kuhla 1995). According to Weissbach and Strubelt (2008), silage DM concentration was corrected for the loss of volatiles during drying.

2.3. Chemical analyses

All samples were kept in a freezer at −18°C until analysis. The fresh forage was freeze-dried and ground over a sieve (1 mm) prior to analysis (Gamma 1–16 LSC, Martin Christ, Osterode, Germany).

Nutrients were determined according to VDLUFA (2012), the official German methods for feed evaluation. Water-soluble carbohydrates were analysed by the anthrone method (von Lengerken and Zimmermann 1991) using a continuous flow analyser (CFA, Scan++, Skalar Analytical, Breda, Netherlands). Determination of organic acids, alcohols and esters was performed in aqueous silage extracts, which were prepared by blending

50 g of the frozen material with 200 ml distilled water and 1 ml toluene. Extracts were kept at 4°C overnight and then filtered through MN 615 filter paper (Machery-Nagel, Düren, Germany), followed by micro-filtration (Minisart RC, 0.45 µm pore size, Sartorius, Göttingen, Germany). The pH was analysed potentiometrically by using a calibrated pH electrode. According to Weiss and Kaiser (1995), lactic acid was detected by high performance liquid chromatography using refractive index detection (LC-20 AB, Shimadzu Deutschland, Duisburg, Germany). Volatile organic acids and alcohols were determined by gas chromatography (GC) using a free fatty acid phase column (Permabond FFAP 0.25 µm, Macherey-Nagel, Düren, Germany) and a flame ionisation detector (FID) (GC-2010, Shimadzu, Deutschland, Duisburg, Germany), as described by Weiss and Sommer (2017). The limit of detection for each parameter was 0.01% of fresh matter. Ethyl esters of lactic and acetic acids were also determined by GC with FID using a 0.25 µm Optima Wax column (Macherey-Nagel, Düren, Germany). Extracts were supplemented with the internal standard 2-methyl pentanol. The detailed description of the method including its precision parameters was published by Weiss and Sommer (2012). The detection limit of esters was determined to be 3 mg/l or 0.001% of fresh matter.

2.4. Microbiological analyses

Fresh maize and maize silage were kept cool until analysis, which was carried out within 4 h after sampling. Microbiological analyses were performed by the accredited laboratory BECIT GmbH, Bitterfeld-Wolfen, Germany. After preparation of serial sample dilutions with peptone water broth (1 g/l), the counts of yeast and moulds were enumerated after spread-plating on yeast extract-dextrose-chloramphenicol agar and incubation for 3 to 5 d at 25°C (ISO 21527, 2008).

2.5. Statistical analysis

The experimental data were analysed separately for each storage time in a framework of a fixed effects model using sealing time (ST) and silage additive (SA) as experimental factors (Milliken and Johnson 2009):

$$y_{ijk} = m + t_i + a_j + (ta)_{ij} + e_{ijk}$$

where y_{ijk} is the observed value of the kth replication from ST i and SA j; μ the population mean; t_i the fixed effect of ST i; a_j the fixed effect of SA j; $(ta)_{ij}$ the fixed interaction effect of both treatment factors; and e_{ijk} the random residual effect of the ijth treatment and kth observation, $\sim N(0,\sigma_e^2)$.

Except for counts of yeasts, normality of observations could be assumed. Main effects and interaction effects were tested by the global F-test and pairwise comparisons between Least Square Means were made by Tukey´s test procedure, considering interactions between both treatment factors. Due to the small sample size, variance homogeneity could not be proven and inferences are based on a common residual variance.

The counts of lactate-assimilating yeasts were $\log_{10}$-transformed before analysis. Values below the detection limit of 100 cfu/g of fresh matter were set at the detection

limit ($\log_{10}$ 2.0). Because of identical observations for several treatments, occasionally under the detection limit, the assumption of normally distributed data could not be supported so that a rank procedure with the ANOVA-type statistical model for the global F test and pairwise comparisons among the rank means was alternatively used.

The relationships between the silage variables (ethanol, lactic acid and acetic acid and their respective ethyl esters as well as ethanol and DM losses) were assessed. The model fit was evaluated by the coefficient of determination (R^2) adjusted for degrees of freedom.

Statistical analysis was performed using MIXED and REG procedures by SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). Statistical significance was declared at $p \leq 0.05$.

3. Results

3.1. Dry matter losses

DM losses, which can be used as a parameter characterising the efficiency of the fermentation process, were always higher in silage sealed with a delay than in promptly sealed silages (Table 2). With the exception of d 3, SA use resulted in lower DM losses compared with untreated silage regardless of ST ($p < 0.001$). The magnitude of the DM loss reduction was consistently larger in silage sealed with a delay than in promptly sealed material (ST x SA interactions $p < 0.05$).

3.2. Production of lactic and acetic acids

Sealing time affected the production of lactic acid in the early stages of fermentation as reflected by lower concentrations caused by delayed sealing, but this effect did not persist during later storage phases (Table 2). The influence of the SA on this organic acid was variable, and interactions between the two tested experimental factors were found on d 16, 34 and 62 of storage. As from d 16 of storage, silage sealed with a delay and treated with SA contained the lowest lactic acid content ($p < 0.05$).

Acetic acid was rapidly formed already during the earlier stages of fermentation (Table 2). Concentrations exceeding 10 g/kg DM, regardless of ST and SA treatment, were detected after three days of fermentation, exceeding 25 g/kg DM in untreated silage at the end of storage. Sealing time increased acetic acid production, and interactions with the factor SA were observed at numerous sampling days.

3.3. Development of yeasts and ethanol production

The development of yeast counts and their major metabolic end product, ethanol, followed a very similar pattern during the early stages of fermentation (Table 2). Up to d 7, higher yeast counts were found in silage than in fresh maize at the time of silo filling (4.3 to 4.4 $\log_{10}$ cfu/g, Table 1), with declining numbers during progressing storage length. On d 3 and 7 of storage, silage sealed with delay had higher yeast counts than promptly sealed material (d 3: $\log_{10}$ cfu/g 7.2 *vs* 5.3, $p < 0.001$; d 7: $\log_{10}$ cfu/g 6.8 *vs* 5.2, $p < 0.001$). The use of SA reduced the yeast population, but the magnitude of the effect

Table 2. Effects of sealing time (ST) (prompt = 0 h *vs*. delay = 24 h) and silage additive (SA) on fermentation characteristics, volatile organic compounds, yeast count and dry matter (DM) losses during the course of maize fermentation (n = 3).

Storage length	Sealing time	SA	Lactate [g/kg DM]	Acetate [g/kg DM]	Ethanol [g/kg DM]	Yeast count [log_{10} cfu/g]	DM loss [%]
3 d	Prompt	-*	14.7^{c}	10.5^{a}	6.1^{a}	5.9^{b}	3.6^{a}
		+♦	13.0^{bc}	11.0^{a}	5.1^{a}	4.7^{a}	3.4^{a}
	Delay	–	10.8^{ab}	12.5^{a}	20.2^{c}	7.3^{d}	10.4^{c}
		+	8.6^{a}	15.6^{b}	16.4^{b}	7.1^{c}	8.2^{b}
	SEM‡		0.70	0.64	0.68	0.03–0.09	0.09
	Effects†						
	SA		0.020	0.020	0.007	<0.001	<0.001
	ST		<0.001	<0.001	<0.001	<0.001	<0.001
	SA × ST		0.708	0.080	0.075	1.000	<0.001
7 d	Prompt	–	24.5^{b}	12.0^{a}	17.7^{b}	6.3^{b}	5.0^{b}
		+	23.4^{b}	12.4^{a}	6.7^{a}	4.1^{a}	3.7^{a}
	Delay	–	18.8^{a}	12.9^{a}	20.7^{bc}	7.1^{c}	10.7^{d}
		+	20.9^{ab}	17.0^{b}	21.8^{c}	6.4^{bc}	8.7^{c}
	SEM		0.81	0.51	0.87	0.03–0.27	0.12
	Effects						
	SA		0.564	0.002	<0.001	0.004	<0.001
	ST		<0.001	<0.001	<0.001	<0.001	<0.001
	SA × ST		0.090	0.007	<0.001	1.000	0.027
16 d	Prompt	–	27.5^{ab}	13.0^{a}	18.5^{b}	3.7^{a}	5.1^{b}
		+	32.7^{b}	12.4^{a}	8.5^{a}	3.8^{ab}	3.9^{a}
	Delay	–	27.1^{a}	13.5^{a}	21.9^{b}	5.5^{b}	11.3^{d}
		+	23.8^{a}	16.3^{b}	19.7^{b}	3.7^{a}	8.9^{c}
	SEM		1.16	0.53	0.89	0 – 0.10	0.09
	Effects						
	SA		0.439	0.069	<0.001	0.065	<0.001
	ST		0.004	0.003	<0.001	0.065	<0.001
	SA × ST		0.006	0.012	0.002	0.022	<0.001
34 d	Prompt	–	31.6^{b}	14.8^{ab}	20.4^{b}	2.7^{a}	5.3^{b}
		+	34.4^{b}	12.5^{a}	8.5^{a}	2.7^{a}	4.0^{a}
	Delay	–	31.4^{ab}	17.4^{bc}	27.9^{c}	2.7^{a}	11.6^{d}
		+	27.5^{a}	19.0^{c}	22.5^{b}	2.7^{a}	9.4^{c}
	SEM		0.88	0.65	0.58	0	0.07
	Effects						
	SA		0.559	0.573	<0.001	1.000	<0.001
	ST		0.004	<0.001	<0.001	1.000	<0.001
	SA × ST		0.005	0.018	<0.001	1.000	<0.001
62 d	Prompt	–	34.0^{b}	16.9^{a}	20.7^{b}	1.7^{a}	5.4^{b}
		+	32.9^{b}	13.9^{a}	8.4^{a}	3.2^{c}	4.0^{a}
	Delay	–	33.6^{b}	20.9^{b}	27.3^{b}	2.6^{b}	11.8^{d}
		+	28.4^{a}	21.6^{b}	21.6^{b}	1.9^{ab}	9.1^{c}
	SEM		0.76	0.75	1.55	0 – 0.10	0.15
	Effects						
	SA		0.003	0.142	<0.001	0.023	<0.001
	ST		0.013	<0.001	<0.001	0.705	<0.001
	SA × ST		0.030	0.038	0.069	<0.001	0.003

(*Continued*)

Table 2. (Continued).

Storage length	Sealing time	SA	Lactate [g/kg DM]	Acetate [g/kg DM]	Ethanol [g/kg DM]	Yeast count [log_{10} cfu/g]	DM loss [%]
142 d	Prompt	–	34.8^{b}	25.2^{b}	20.8^{b}	2.2^{a}	6.0^{b}
		+	34.0^{b}	15.2^{a}	8.3^{a}	1.7^{a}	4.1^{a}
	Delay	–	33.4^{b}	27.9^{b}	32.3^{c}	1.9^{a}	11.9^{d}
		+	28.6^{a}	24.6^{b}	17.7^{b}	2.0^{a}	8.9^{c}
	SEM		0.89	1.08	0.75	0 – 0.54	0.16
	Effects						
	SA		0.014	<0.001	<0.001	0.364	<0.001
	ST		0.005	<0.001	<0.001	0.452	<0.001
	SA × ST		0.055	0.013	0.197	0.732	0.008

*-, untreated; ♦+, treated with chemical additive composed per litre of 257 g sodium benzoate, 134 g potassium sorbate, 57 g ammonium propionate, applied at 2 ml/kg; † p values of global F-test or non-parametric global test, least-square means in columns within storage length differ if they have no common superscript ($p < 0.05$; Tukey`s test or for yeast count non-parametric rank test); ‡SEM, standard error of means (for yeast count based on individual treatments, for other variables based on residual variance).

was larger in delayed than in promptly sealed silage (d 3: log_{10} cfu/g 6.6 *vs* 5.9, $p < 0.001$; d 7: log_{10} cfu/g 6.7 vs 5.3, $p < 0.004$).

Concurrently, high ethanol concentrations above 10 g/kg DM had already accumulated during the first 16 d of storage in treatments CON, CON_delay and SA_delay. As from d 7, promptly sealed silage treated with SA consistently had the lowest ethanol content (≤8.5 g/kg DM, $p < 0.001$), which remained stable until the end of fermentation. A strongly positive, linear correlation was detected between the concentration of ethanol and the DM losses during fermentation ($R^2 = 0.70$, Root MSE = 1.66, $p < 0.001$, Figure 1).

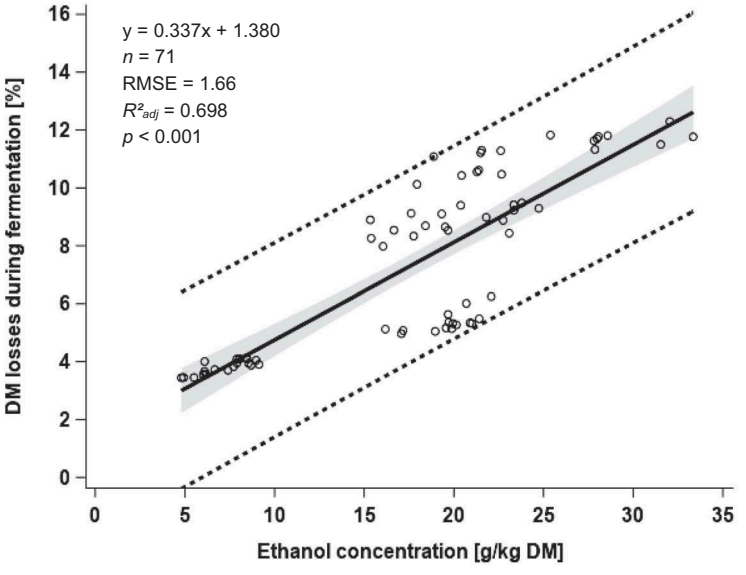

Figure 1. Relationship between ethanol concentration and dry matter (DM) losses during fermentation of maize silage.

3.4. Formation of ethyl esters

The formation of ethyl lactate (EL) persisted throughout the fermentation process (Figure 2). At the end of storage, CON_delay silage contained the highest level (508 *vs* 194 … 334 mg/kg DM, $p < 0.001$) and SA use largely restricted the EL accumulation (prompt: 194 *vs* 334 mg/kg DM, delay: 264 *vs* 508 mg/kg DM, $p < 0.001$). On the contrary, ethyl acetate (EA) was produced very rapidly and intensively (Figure 3) reaching concentrations of about 3000 mg/kg DM in CON_delay silage already after 3 d of storage. On d 34, the highest level was measured in untreated silage sealed with delay (4556 mg/kg DM) compared with the other treatments (1018 to 2314 mg/kg DM, SA × ST interaction, $p < 0.001$). Similar EA accumulation pattern was observed over time in all treatments, but the analysed concentrations differed depending on storage length. The treatment with SA consistently reduced EA formation in silages sealed with a delay regardless of sampling day, but the magnitude of the effect differed widely (ST × SA interactions, $p < 0.01$ or smaller).

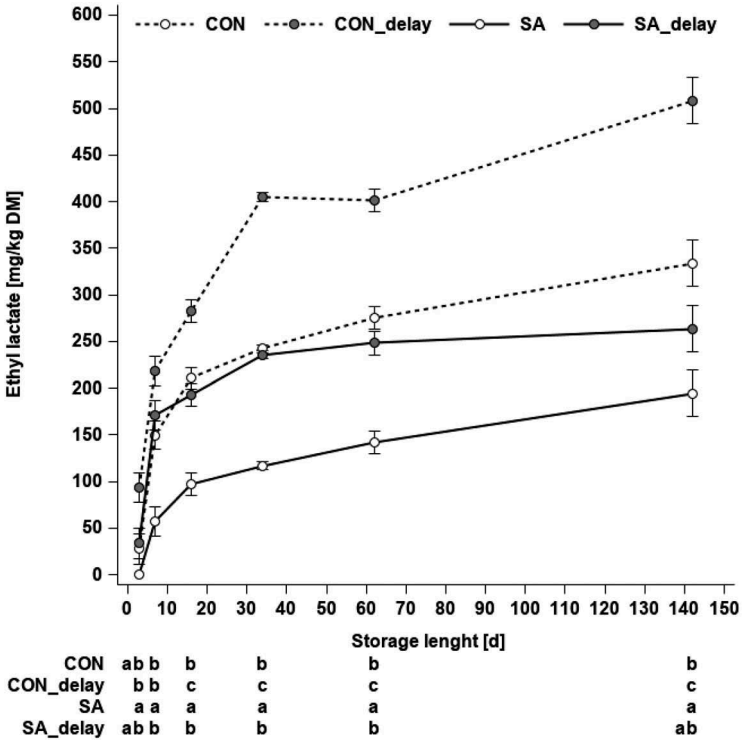

Figure 2. Changes in the concentration of ethyl lactate during fermentation of maize silage as affected by a silage additive containing sodium benzoate, potassium sorbate and ammonium propionate and prompt or delayed sealing.

Least square means are presented with standard error of means as error bars, significant differences within respective storage length are given below as plot table (means with no common letter differ at $p < 0.05$; Tukey`s test).CON, control, promptly sealed; SA, with silage additive, promptly sealed; CON_delay, control, sealed with 24 h delay; SA_delay, with silage additive, sealed with 24 h delay.

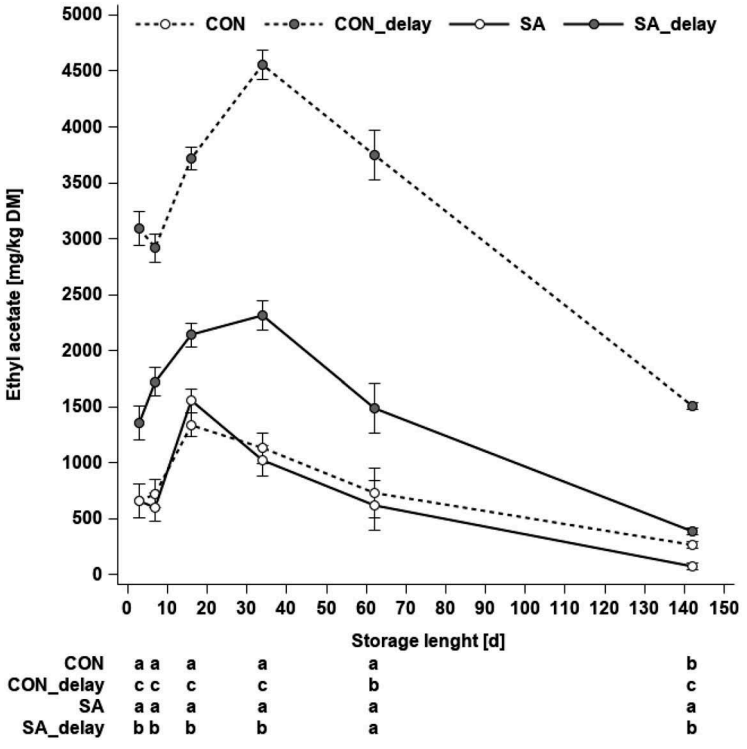

Figure 3. Changes in the concentration of ethyl acetate during fermentation of maize silage as affected by a silage additive (containing sodium benzoate, potassium sorbate and ammonium propionate) and prompt or delayed sealing.

3.5. Relationships between the concentrations of ethanol and ethyl esters

Between the concentrations of ethanol and its esters of lactic and acetic acids positive linear relationships were observed (Figure 4). However, the correlation between the concentrations of ethanol and EL (R^2 = 0.65, Root MSE = 74.8, $p < 0.001$) was stronger than between ethanol and EA (R^2 = 0.35, Root MSE = 985.1, $p < 0.001$).

4. Discussion

4.1. Formation of respective organic acids lactic and acetic acid for ester production in silages

Regardless of SA use, in this study the lactic acid content of mature maize silage (stored for more than 4 weeks) was detected at considerably lower concentrations than found for maize silage of similar DM level (<30%) (Kung et al. 2018; Brüning et al. 2018a). On the contrary, acetic acid production was similar as observed by these authors. As maize fermentation is a complex process involving many different microorganisms performing a range of metabolic pathways, the production of organic acids may be very different even if the DM content at ensiling is similar. Delayed sealing reduced lactic acid production, as described by Brüning et al. (2018a) and increased acetic acid production (Mills and Kung

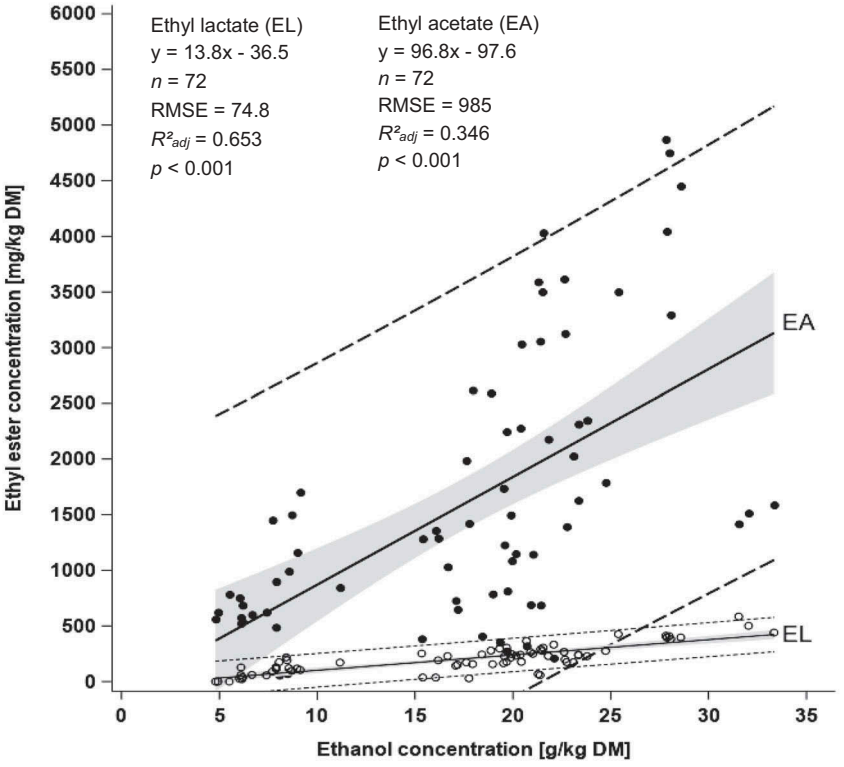

Figure 4. Relationship between the concentrations of ethanol and ethyl esters of acetic and lactic acids in maize silage.

○ Ethyl lactate (EL), ● Ethyl acetate (EA)

2002), but interactions with SA treatment were frequently found. This may be associated to potentially selective effects of the SA on certain acetate-producing microorganisms, which may be active in different phases of the fermentation process (Pahlow et al. 2003).

4.2. Formation of ethanol

Ethanol was rapidly formed already during the initial stages of fermentation, reaching a concentration of >5 g/kg DM after 3 d and the highest value of 32.3 g/kg DM after 142 d of fermentation. This was much higher than previously reported in maize silage stored for 141 d (Brüning et al. 2018a). A stimulation of ethanol production by delayed sealing was already observed during the early storage period. This is in line with data on whole-crop barley silage sampled after 60 d of storage (Mills and Kung 2002), but contradicts other results on maize silage, where no effect of ST after extended storage periods (>90 d) was detected (Weiss et al. 2016; Brüning et al. 2018a). In our study, the effect of ST persisted, but an interaction with the factor SA was frequently observed on several sampling days. Promptly sealed maize silage treated with SA consistently had the lowest ethanol content and DM losses. Our results on the power of the relationship between ethanol content and DM losses agree very well with data on sugarcane (Rabelo et al. 2018), confirming that

ethanol-forming metabolic pathways are the most substantial sources of DM losses during fermentation. As there is a range of microorganism groups capable of producing ethanol, including yeasts and heterofermentative LAB (McDonald et al. 1991), it can only be hypothesised which population contributed most to the total concentration of this alcohol.

Considering the data on the yeast counts, which showed a dramatic increase up to d 7 of storage in all treatments except promptly sealed, SA treated maize silage, it seems very likely that the major fraction of the total ethanol concentrations can be attributed to yeast metabolism. However, this may seem contradictory regarding silages exposed to air for 24 h before sealing because ethanol is produced by yeasts under anaerobic conditions. It can be assumed that oxygen was rapidly depleted after sealing, causing yeasts to switch their metabolism from respiration to fermentation. Furthermore, as the whole content of each silo was thoroughly mixed before sampling, it seems reasonable to believe that, due to high compaction, air mostly affected the upper silage layers and that a fermentation had already begun in the lower sections of the silos. Thus, potential differences in composition between silage layers could not be detected.

4.3. Production pathways of ethyl acetate and ethyl lactate in silages

Ethyl esters of lactic and acetic acids were already detected during the initial stages of storage, but their final concentrations and their accumulation pattern differed largely between the substances. EL showed an increase over the entire storage period with the highest concentration analysed at the termination of the trial after 142 d. The final EL content was similar to data presented by Weiss (2017). In contrast to these experiments, EA concentrations up to 10-fold higher than EL were found in the present study. Higher values of EA in comparison to EL were also reported by Gerlach et al. (2013) and Brüning et al. (2018a), but the maximum ratio between these two compounds was lower. The power of the relationship between ethanol and EL was comparable to results of Weiss et al. (2016), whereas the relationship between ethanol and EA was characterised by smaller coefficients of determination than previously reported (Weiss et al. 2016). This discrepancy can be explained by different accumulation pattern of the reactive substances. Ethanol concentrations increased during the course of fermentation, whereas ethyl acetate decreased after it had peaked on d 34. In all studies, which have yet described the relationships between ethanol and ethyl esters were based on single-point evaluations. Considering only data of the present study measured at d 142 of storage, the coefficients of determination were very similar to those reported in the literature (EA: $R^2 = 0.80$, RMSE = 261.0, $p < 0.001$; EL: $R^2 = 0.85$, RMSE = 48.7, $p < 0.001$).

The similar pattern of ethanol and EL formation during the early period of storage supports the assumption that EL is formed by chemical synthesis as repeatedly suggested by Weiss et al. (2016). The chemical reaction of lactic acid with ethanol in the presence of charged hydrogen ions takes place slowly and the equilibrium changes to ester formation in the presence of a surplus of reactants (Peter and Vollhardt 1988). This hypothesis was confirmed by this study showing only marginal concentrations of EL after 3 d of fermentation, and by Brüning et al. (2018a), who could not detect EL 2 d post-filling before sealing of 120-l drums.

The present study showed an intensive and parallel increase of the EA level and yeast counts especially in delayed sealed silages during the first 3 d of fermentation, supporting data reported by Brüning et al. (2018a). Because certain yeast species have the capacity to synthesise

EA (Nordström 1966; Fredlund et al. 2004; Park et al. 2009; Kruis et al. 2017), this rapid increase in EA concentration can be attributed to the catalytic action of enzymes present in yeasts. According to Park et al. (2009), three types of enzymes have previously been associated with EA formation in yeast: esterases, hemiacetal dehydrogenases (HADH) and alcohol acetyl transferases (AAT). Intracellular esterases are responsible for the hydrolysis of EA in the presence of water to acetate and ethanol. AAT catalyses the formation of EA from acetyl-CoA and ethanol, and HADH the formation of EA from reduced hemiacetals like ethanol and aldehyde (Kruis et al. 2017). The equilibrium reactions depend on numerous factors such as acetate concentration in yeast cells to that of acetyl-CoA (Schermers et al. 1976), pH and available carbohydrates (Yoshioka and Hashimoto 1984).

The decline in EA concentration despite the increase in the contents of the reactants ethanol and acetic acid during the fermentation process may be also attributed to the EA hydrolysis in aqueous systems. Water was sufficiently present in the silage due to the high initial moisture content of the forage of about 73%, which further increased during storage caused by high DM losses, especially in silage sealed with a delay.

An alternative explanation for the observed differences in accumulation pattern between the ethyl esters may be provided based on the vapour pressure of the substances, which is much higher for EA (98 mbar) than for EL (2 mbar). Thus, more than the produced EA may have escaped the porous silage and collected in the headspace of the jars to be released along with other fermentation gas whenever a lid-lifting overpressure had built-up.

4.4. Effects of additives containing sorbate and benzoate on ethanol and ethyl ester concentration

It is well known that SA can alter ethanol production, thereby exerting an effect also on ethyl ester content. The decreased concentrations of ethanol and ethyl esters in this study, especially in promptly sealed silages, may be associated to the strong inhibitory effect of sorbate, benzoate and propionate on yeasts (Woolford 1975) as the most prominent ethanol producers in silage (McDonald et al. 1991). The results support previous findings on silages made from maize, whole-crop cereals and sorghum (Weiss 2017), which revealed a substantial restriction of ethanol and ethyl ester formation by treatment with a combination of sodium benzoate and potassium sorbate, or by the sole use of potassium sorbate (Hafner et al. 2014, 2015).

5. Conclusions

Delayed sealing stimulated yeast activity, resulted in excessive ethanol production and the formation of ethyl esters of lactic and acetic acids, resulting in high DM losses. The correlation between the concentrations of ethanol and ethyl esters varied depending on the type of ester providing evidence of differing production pathways. From the different concentration and accumulation patterns between EL and EA, especially during the early fermentation phases, the conclusion is drawn that the major proportion of EA was produced directly by biochemical pathways, whereas EL was primarily formed by chemical reaction of ethanol and lactic acid. The use of the chemical SA containing active ingredients with specific antifungal effects can reduce the ethanol and VOC production significantly, thereby reducing DM losses regardless of storage conditions.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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